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Cloning and Expression of Genes for Dengue Virus  
Type 2 Encoded Antigens for Rapid Diagnosis and Vaccine Development

ANNUAL PROGRESS REPORT

by

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<p>1. We verified the DNA sequences of two cDNA clones of Dengue virus RNA by determining their nucleotide sequences from the 5' end. We discovered some errors (ten single nucleotide changes) in our previous sequence data comprising 2677 nucleotides. The verified sequence was submitted for publication (Yaegashi et al., 1985) which is in press (Gene, 1986).</p> <p>2. We chemically synthesized specific oligodeoxynucleotide primers based on our sequence data. Primer #1 - #3 represent oligodeoxynucleotides (see attached page)</p>					
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from pVV9, pVV17 and 3' end of Dengue Virus RNA. The specificity of two of these primers was verified by hybridization of 5' labeled oligonucleotides used as probes to the cDNA inserts released from pVV9 and pVV17 DNA by PstI digestion. We showed that Primer #1 hybridized specifically with pVV9 cDNA and Primer #2 with pVV17 cDNA.

3. More cDNA clones of DEN-2 RNA were generated using calf thymus random primers for the reverse transcriptase-catalyzed reaction. The double-stranded cDNA was cloned in three ways; i) by oligo(dC)-tailing, followed by annealing to oligo(dG)-tailed vector and transformation, ii) by ligation to SalI linkers, followed by cleavage with SalI and cloning at the SalI site of pUC18 vector DNA, iii) by treatment with EcoRI DNA methylase, ligation to EcoRI and cloning at the EcoRI site of pUC18 vector DNA. A total of 74 clones with inserts ranging in size from 700 bp - 2 kb and one 4 kb long clone were obtained. The dengue viral specificity of these clones were examined by hybridization with the cDNA probe synthesized from dengue RNA template and random calf thymus DNA primers.
4. We have cloned the cDNA from region II between pVV1 and pVV17 cDNA clones by using a restriction fragment, obtained by cleavage of pVV17 DNA with EcoRI + NcoI, as primer from cDNA synthesis. This cDNA, 2 kb in length hybridizes with pVV1 and pVV17 cDNA as probes as expected. The sequence analysis of this clone is in progress.
5. Using our Primer #1, Dr. Robert Putnak of Walter Reed Army Institute of Research cloned the complete NS1 coding region using pUC18 vector. We are sequencing two independent clones, pRP2 and pRP28 by Maxam and Gilbert's chemical sequencing method.
6. Based on our sequence data, we chose two hydrophilic regions from NS1 and NS5 coding regions and synthesized two peptides to raise NS1 and NS5 specific antibodies by scientists at Walter Reed Army Institute of Research.

## FOREWARD

The investigators have abided by the National Institutes of Health Guidelines for Research involving Recombinant DNA molecules (April 82) and the Administrative Practices Supplements, as indicated in the Memorandum of Understanding and Agreement, approved by Institutional Biosafety Committee and N.I.H.

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## INTRODUCTION

This PROGRESS REPORT (September 15, 1985 - September 14, 1986) can be divided into five parts focusing on the overall goal of determining the complete sequence analysis of dengue virus genome, the expression of the cloned genes, especially the gene for the structural surface glycoprotein E in E. coli and characterization of the dengue viral antigens synthesized in the infected cell using the antibodies raised against the different polypeptide segments of the dengue viral polyprotein, expressed in E.coli.

1. Verification of the DNA sequence data previously generated for pVV1 and pVV9 clones, mapping in the region of dengue virus genome coding for non-structural proteins.
2. Chemical synthesis of oligodeoxynucleotide primers, specific for the region encoded in pVV9, pVV17 and 3' end of the dengue virus genome. Verification of these primers for the specificity by hybridization with pVV1 and pVV9 clones.
3. Synthesis of cDNA copy of the dengue virus type 2 RNA in a reverse transcriptase catalyzed reaction, followed by double-stranded cDNA synthesis and transformation of E.coli JM83/pUC18 host/vector system.
4. Screening of the recombinant cDNA clones by restriction enzyme analysis and by Southern hybridization.
5. Synthesis and characterization of short peptide antigens, representing short hydrophilic segments of dengue viral proteins, the amino acid sequences of which were deduced by our DNA sequence data. (AU).

1. Verification of the DNA sequence data previously generated for pVV1 and pVV9 clones.

a. Rationale

In the Annual Report dated March 1986 covering the period, September 1, 1984 - August 31, 1985, for the project entitled, "Structure and Functional Studies on Dengue-2 Virus Genome" for Contract No. DAMD 17-82-C-2051, I reported the nucleotide sequence data of three cDNA clones, pVV1, 9, and 17 of DEN-2 RNA. These clones were sequenced by using the Maxam and Gilbert's (1) procedure. The data of pVV17 clone was obtained by sequencing from the 3' end of overlapping subclones generated by BAL-31 nuclease. The data were verified by sequencing the complementary 5' strand of these clones up to 75% of the total (2 kb) length of pVV17. However, the nucleotide sequence data of DEN-2 cDNA clones, pVV1 and 9 were sequenced only from the 3' end of one strand. In order to obtain unambiguous sequence data, both strands of DNA must be sequenced. Many scientific journals insist on this criterion for the reliability of the data before accepting for publication. While sequencing an independent DEN cDNA clone, pVV18, which has a 1.75 kb cDNA insert and overlaps with pVV9, we noticed a few discrepancies, all being single nucleotide substitutions. Therefore, we undertook the sequence analysis of the subclones of pVV' and 9 from the 5' end in order to get unambiguous data. In some regions of pVV9, we used the overlapping, pVV18 cDNA clone. The results are reported in this section.

b. Experimental.

The plasmid DNA (20  $\mu$ g) from the parent clones, pVV9, pVV1 or pVV18 or subclones of either pVV9 or pVV1 was digested with BamHI (25 units) at 37°C for 60 min. After complete linearization, the plasmid DNA was dephosphorylated by calf-intestinal alkaline phosphatase and labeled at the 5' end using polynucleotide kinase according to Maniatis et al. (1982). The 5' labeled DNA was sequenced according to

Maxam and Gilbert (1977). The complete nucleotide sequence of complementary strand of overlapping clones, pVV1 and pVV9 was obtained. We discovered a few errors in our previous data reported in ANNUAL REPORT dated March 1986 for DAMD17-82-C-2051 and the corrected data were incorporated in the manuscript by Yaegashi et al. (1986). The sequence data shown in Fig. 2 show the revised sequence.

## 2.2 Chemical synthesis of short oligodeoxynucleotide primers specific for pVV9, pVV17 and for the highly conserved sequence found at the 3' end of other flaviviruses.

### a. Rationale.

Fig. 1 shows the alignment map of our cDNA clones pVV1, pVV9, pVV17 and pVV18 on the dengue virus type 2 genome. In accordance with the description/specifications/work statement of the contract, we initiated cDNA synthesis for the entire genome of Dengue-2 virus. The approach we had taken to use synthetic DNA primers whose sequences were selected based on our knowledge of the nucleotide sequence of the cDNA clones which we had already determined (see Fig. 2 of this report and Fig. 6b of ANNUAL REPORT dated March 1986 for Contract No. DAMD17-82-C-2051. Our alignment of the nucleotide sequence of Dengue virus cDNA clones with the published data of yellow fever virus genome of Strauss and coworkers revealed that the pVV9 clone is situated most 5' of all cDNA clones (except pVV18 which has 500 bp extra nucleotides towards the 5' end of pVV9 for which we have not completed the sequence analysis) (Fig. 1). From these data, we chose to synthesize two DNA primers (PRIMER #1 and PRIMER #2, see below), #1 being specific to pVV9 and PRIMER #2 specific to pVV17. These DNA primers could be used to synthesize cDNA in a reverse transcriptase catalyzed reaction to copy region I and region II of Dengue virus RNA (Fig. 1). In order to copy the region III of dengue virus type 2 RNA, the following strategy was used.

The extreme 5' and 3' terminal sequences of yellow fever virus (Rice et al. 1985) and West Nile virus (Wengler and Wengler, 1981) are homologous (see Fig. 3). Using this conserved sequence data, we synthesized a DNA primer which is 14 nucleotides long (PRIMER #3) for use to copy region III (Fig. 1). We verified the specificity of these primers by Southern hybridization with pVV9 and pVV17 cDNA inserts. These two primers #1 and #2 hybridized specifically to pVV9 and pVV17 cDNAs, respectively (See Experimental).

b. Experimental

The synthesis of the primers were carried out by phosphate triester chemistry in an automated DNA synthesizer (BioSearch, San Rafael, CA). The final products, covalently attached to the silica gel were released and the protecting groups on the purines and pyrimidines of the primers were removed and purified as follows:

1. The dry silica gel containing the covalently attached primers were treated with 1 ml of concentrated ammonium hydroxide in a polypropylene tube with a screw cap and the contents were vortexed briefly. The tubes were incubated for 3 hours at room temperature with cap securely fastened. This step will detach the oligomer from the silica support.
2. The tubes were vortexed again and centrifuged for 1 min and pipet the  $\text{NH}_4\text{OH}$  solution into another Eppendorf tube. The tubes were closed tightly and then incubated at  $55^\circ\text{C}$  overnight. This step thoroughly deblocks all the protecting aromatic groups from the oligomers.
3. The contents were centrifuged and the supernatant was evaporated to dryness in a high-speed vacuum desiccator (Savant Instruments). The crude product is now ready for purification. The primers were purified by electrophoresis on polyacrylamide gels. The deblocked, dry product

from a synthesis run was taken (25% of the material) in an Eppendorf tube and 50  $\mu$ l of water was added. The sample was heated to 50°C for 2 min to dissolve the contents and centrifuged to remove any insoluble matter. Sample loading dye was added containing formamide, (50%), Xylene cyanol and bromophenol blue (0.03% each). The contents were heated at 90°C for 5 min. and quick-chilled. The samples were loaded on a 25% polyacrylamide gel (22 x 40 cm) and electrophoresed for 7 hours at 500 V. The gel was placed on a Saran wrap and visualized by UV shadowing technique. In a darkroom, the gel was placed on a TLC plate which contains a fluorescence indicator and short wavelength UV was used to visualize fluorescence quenching. The bands containing the desired oligonucleotides were cut out and sliced into small pieces. These pieces were soaked in 0.1 M  $\text{NH}_4\text{HCO}_3$  and incubated at 50°C for 10 min and then at room temperature overnight. The supernatant was transferred to a second tube and the elution of the oligomer from the gel was repeated once more. The combined supernatants were evaporated to dryness, desalted by resuspending it in water and by Sephadex G-25 column chromatography. The eluates in 0.001 M Tris. HCl, pH 7.5 and .0001 M EDTA were lyophilized and stored in 0.01 M Tris.HCl and .001 M EDTA. The concentrations were estimated by absorbance at 260 nm.

The specificity of oligonucleotides, #1 and #2 were verified by hybridization with pVV9 cDNA insert for #1 and pVV17 for #2. The pVV9 and pVV17 plasmids were cut, with Pst I and the digests were fractionated on an agarose gel. The gel was stained with ethidium bromide and photographed (Figs, 4b and 5b), lane 1, pVV17 + Pst I; lane 2, pVV9 + Pst I). The DNA from the gels were transferred to the Gene Screen Plus and the membranes were hybridized with 5'-labeled pVV9-specific primer (#1) (Fig. 4a) and

pVV17-specific primer (#2) (Fig. 5a). As shown in Figs. 4 and 5, primer #1 hybridized specifically with 1.6 kb long, pVV9 cDNA insert released by Pst I (Fig. 4a, lane 2), and primer #2 hybridized specifically with 2 kb long pVV17 cDNA insert released by Pst I (Fig. 5a, lane 1). Thus, the primers 1 and 2 are suitable as primers for reverse transcriptase-catalyzed cDNA synthesis and cloning and double-stranded cDNA. Their use for cDNA cloning is described in the following section.

### 2.3. Synthesis of cDNA copy of the Dengue virus type 2 RNA and cloning cDNA in E.coli JM83/pUC18 host vector system

a. Rationale. In order to determine the complete nucleotide sequence of the regions I, II and III of Dengue virus RNA (Fig. 1), it was necessary to obtain additional cDNA clones from these regions either by random priming approach (Taylor et al., 1976; Rice et al., 1981) or specific oligonucleotide priming approach. Using the random priming approach the double-stranded (ds) cDNA obtained by using the method of Gubler and Hoffman (1983) was oligo d(C)-tailed in one experiment and was ligated to SalI-linker in another experiment, followed by digestion with SalI. The oligo d(C)-tailed ds cDNA was annealed to oligo d(G)-tailed pUC18 DNA and the annealed mixture was used to transform E.coli JM83. The ds cDNA containing SalI site at both ends was cloned at the SalI site of pUC18 using E.coli JM83 as the host. The ds cDNA synthesized using random primers was also treated with EcoRI methylase to make all the internal EcoRI sites of cDNA resistant to EcoRI by methylation so that synthetic EcoRI site can be created at the termini by ligation of linkers, followed by digestion with EcoRI. This cDNA was cloned at the EcoRI site of pUC18 using E.coli JM83 as host. The transformants were selected on an agar plate containing IPTG + X-gal and the white colonies were screened for inserts by restriction enzyme analysis and Southern hybridization using labeled DEN-2 cDNA as probe.

b. Experimental. The strategy used for cDNA synthesis using DEN-2 RNA template, a mixture of oligo d(T) and calf thymus random primers (Fig. 6) is shown.

2.3.1. cDNA synthesis by random primers. DEN-2 RNA was denatured by incubating the RNA (5  $\mu$ g) with 1  $\mu$ l of methylmercuric-hydroxide (100 mM) at room temperature for 10 min. Excess of the denaturant was neutralized by incubating the above mixture with 1.5  $\mu$ l of  $\beta$ -mercaptoethanol (1M), followed by the addition of RNasin (RNase inhibitor) (25 units) for 5 min at room temperature. The first strand cDNA synthesis was carried out by using a mixture of oligo d(T) (2.8  $\mu$ g), calf thymus DNA primers (5  $\mu$ g), 50 mM Tris-HCl pH 8.3, 10 mM  $MgCl_2$ , 0.14M KCl, a mixture of all 4 dNTP [400  $\mu$ M each], 50  $\mu$ Ci of dCTP ( $\alpha$ - $^{32}P$ ) and reverse transcriptase from avian myeloblastosis virus (90 units). Incubation was at 41°C for 3 hrs. Aliquots (1  $\mu$ l) of 0' and 180' were withdrawn and were analyzed for acid-insoluble radioactivity incorporated into the first strand of cDNA synthesized. The reaction was stopped by the addition of EDTA to 20 mM. The cDNA was extracted with phenol:  $CHCl_3$  mixture, and then with ether (2x) and precipitated with ethanol. The pellet was resuspended in 50  $\mu$ l of 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA and then 50  $\mu$ l of 4 M ammonium acetate was added. After mixing, 100  $\mu$ l of ethanol was added. The ethanol precipitate of cDNA was collected by centrifugation and the pellet was dried.

2.3.2. Second-strand synthesis of cDNA. Synthesis of second-strand cDNA was carried out by incubating a reaction mixture (100  $\mu$ l) sequentially at 12°C and 22°C for 60 min each. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 5 mM  $MgCl_2$ , 10 mM  $(NH_4)_2SO_4$ , 100 mM KCl, 0.15 mM  $\beta$ -NAD, 50  $\mu$ l/ml BSA, 40  $\mu$ M dNTPs, 8.5 units/ml of E.coli RNase H, 230 units/ml of E.coli DNA polymerase I, and 10 units/ml of E.coli DNA ligase. The reaction was stopped by the addition of EDTA to 20 mM. The double-stranded cDNA was recovered by phenol extraction, ethanol precipitation and centrifugation to get the pellet. The pellet was washed with 80% ethanol and dried in

vacuum. It is resuspended in TE, pH 7.5 and analyzed for the size of alkaline agarose gel electrophoresis (Fig. 7). It was heterogeneous in size between 500 bp -> 2 kb in length.

2.3.3. Conversion of ragged ends of ds cDNA to blunt ends. The ds cDNAs obtained by random primers (or by synthetic primers) are not all likely to contain blunt ends. Therefore, it was necessary to treat them with Klenow's DNA polymerase to convert 3' or 5' protruding ends into blunt ends. It was carried out in a reaction mixture (30  $\mu$ l) containing 130  $\mu$ M dNTP and 10 units of Klenow DNA polymerase in nick-translation buffer. The reaction was carried out for 30 min. at room temp. Then, the ds cDNA was purified by NEN SORB (NEN-DuPont, Boston, MA) affinity column and the 50% methanol eluate was vacuum dried.

2.3.4. Oligo(dC)-tailing of ds cDNA or oligo(dG)-tailing of PstI-linear pUC18 DNA using terminal transferase. Approximately 500 ng of cDNA (or 1  $\mu$ g of pUC18 DNA linearized by PstI) was incubated in 50  $\mu$ l containing 100 mM Na-cacodylate (pH 6.9), 1 mM CoCl<sub>2</sub>, 0.2 mM DTT, 100  $\mu$ M dCTP + 10  $\mu$ Ci dCTP ( $\alpha$ -<sup>32</sup>P) (100  $\mu$ M dGTP in the case of Pst I linear of pUC18 DNA + <sup>3</sup>H-dGTP for oligo(dG)-tailing) and 17 units of terminal transferase. The reaction was carried out at 37°C for 30 min.

2.3.5. Transformation of E.coli JM83 with annealed mixture of oligo(dC)-tailed cDNA. E.coli JM83 were made competent for transformation following the procedure of Hanahan (1983). This procedure is at least two orders of magnitude higher in transformation efficiency compared to other procedures. Oligo(dG)-tailed pUC18 DNA (90ng) was mixed with DEN2 cDNA (45 ng) in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl. The mixture was heated at 65°C for 5 min, incubated at 57°C for 2 hr and then at room temperature for 2 hr. Competent cells (0.2 ml) of E.coli were added to the annealed mixture as well as to appropriate control DNAs. The mixture was incubated in ice for 30 min and then at 42°C for 90 sec. Then, 2X YT

broth (1 ml) was added to each tube and tubes were incubated at 37°C for 1 hr. Aliquots were plated on YT agar plates containing 50 µg/ml ampicillin, 40 µg/ml IPTG and 40 µg/ml of X-gal (Sigma Chemical Co., St. Louis, MO). The agar plates were incubated at 37°C overnight for the transformed colonies to grow. A total of 100 colonies were picked for screening by mini plasmid preparation and restriction enzyme analysis.

2.3.6. Screening of the transformants by miniplasmid isolation. The plasmid DNA was isolated from each of the colonies picked by the mini plasmid preparation method as described in Maniatis et al. (1982). The procedure followed involved briefly treatment with a mixture of alkali and SDS, followed by a selective precipitation of E.coli chromosomal DNA by K. acetate, pH 5.7. The plasmid DNA from the supernatant was extracted with phenol/CHCl<sub>3</sub> mixture followed by ether extraction and ethanol precipitation. The pellet of DNA was resuspended in TE buffer (10 mM Tris HCl, pH 7.5 and 1 mM EDTA) containing 20 µg/ml RNase A.

The screening of the plasmid DNAs for an insert was carried out by digestion with Pst I. The digests were fractionated on an agarose gel (1%), out of 100 clones examined, 38 colonies had inserts, majority of them had insert sizes between 500-700 bp. However, 5 colonies had insert sizes ranging from 1.3-2 kb long.

2.3.7. Fractionation of ds cDNA from section 2.3.3. by agarose gel electrophoresis. Only one fourth of the ds cDNA was used for oligo(dC)-tailing reaction described in Section 3.4. The remainder of the cDNA was fractionated to size the cDNA molecules on a low melting agarose (LMP-agarose). The gel pieces representing 500 bp-2 kb and 2 kb - 5 kb were cut out and eluted. The gel piece containing cDNA of 2 kb or more was used for oligo(dC)-tailing or ligation to SalI-linkers as described below.

2.3.8. Oligo(dC)-tailing of ds cDNA (> 2 kb fraction). The conditions for oligo(dC)-tailing, followed by annealing with oligo(dG)-tailed vector DNA and transformation was as described in the previous sections. The transformation did not result in anymore useful transformants than the experiment described in Section 3.5, possibly due to the dG-tailed vector was contaminated with traces of supercoiled (PstI-uncut) vector which gave rise to a large background of blue colonies with no DNA insert.

2.3.9. Ligation of cDNA to synthetic SalI linker. Two  $\mu\text{g}$  of synthetic SalI linker (NEBL, Beverly, MA) was phosphorylated using T4 polynucleotide kinase as described by Maniatis et al. (1982). The phosphorylated linker (0.8  $\mu\text{g}$ ) was ligated to cDNA (500 ng) overnight and the mixture was extracted with phenol/ $\text{CHCl}_3$  and ether. It was then precipitated with ethanol. The pellet was resuspended in high salt restriction buffer and digested with SalI (100 units) for 3 hours at  $37^\circ\text{C}$ .

The vector DNA (pUC18) was prepared by digestion with SalI to linearize the plasmid. The vector DNA and the cDNA were fractionated on a LMP-agarose gel. The vector DNA was ligated to cDNA and was used to transform JM83 cells. The transformants were plated on amp/IPTG/X-gal agar plates. Fortysix white colonies were picked for screening by miniplasmid preparation and restriction enzyme analysis by cutting with SalI to release the cDNA insert, followed by size analysis by agarose gel electrophoresis. Twelve clones had insert sizes between 0.5 kb- 1 kb in length and ten clones had insert sizes between 1 kb - 2 kb and one clone had cDNA insert of about 4 kb in length.

2.3.10. Treatment of DEN-2 cDNA with EcoRI methylase, followed by ligation to EcoRI linkers and digestion with EcoRI. DEN-2 cDNA (500 ng) was treated with EcoRI DNA methylase in a reaction mixture (100  $\mu\text{l}$ ) containing 100 mM Tris.HCl pH 8.0, 10 mM EDTA, 1 mM S-adenosyl methionine and EcoRI DNA methylase. The reaction was incubated at  $37^\circ\text{C}$  for 1 hr. The cDNA was then extracted with phenol/ $\text{CHCl}_3$  and

ether and then precipitated with ethanol. The pellet after centrifugation was resuspended in 11  $\mu$ l of TE buffer. It was then ligated with 50 ng of phosphorylated EcoRI linker in a reaction mixture containing 2 units of T4 DNA ligase. It was then purified by LMP-agarose gel to remove the excess free EcoRI linkers. The cDNA ligated to EcoRI linkers were eluted from the gel and ethanol precipitated. It was then cloned at the EcoRI site of pUC18 vector and the mixture was used to transform E.coli JM83. Twenty white colonies were picked from amp/IPTG/x-gal agar plates. In all the experiments described above, the DEN-2 cDNA was synthesized using calf thymus random DNA primers. The ds cDNA was cloned at the PstI site of pUC18 DNA by either G-C tailing method or at the SalI site or EcoRI site of pUC 18 DNA. These colonies were screened by restriction enzyme analysis for the presence or absence of inserts and then by hybridization to ( $^{32}$ P)-labeled cDNA probe synthesized from DEN-2 RNA template.

2.3.11. Screening of the transformants by restriction enzyme analysis of miniplasmid preparations and by Southern hybridization. The transformants obtained by oligo(dG)-oligo(dC) tailing method (Section 3.5 and 3.6), SalI linker ligation method (Section ix), or EcoRI linker ligation method (Section 3.10) were initially screened by restriction enzyme analysis by cutting with PstI, SalI or EcoRI, respectively. The digests were fractionated by agarose gel electrophoresis and the DNA fragments were transferred to NEN-Gene Screen Plus membrane filter. The DNA immobilized onto the filter was hybridized with the DEN-2 cDNA prepared from DEN-2 RNA using calf thymus random primers. The conditions for preparing the probe are described in annual progress report dated September 14, 1983 for Contract No. DAMD17-85-C-5273.

The restriction cleavage patterns of the clones generated by oligo(dC):oligo(dG) tailing method are shown in Figs. 8 and 9. Fig. 9 also contains one clone which was generated by EcoRI linker-ligation method (lane 12, Fig. 9). Fig. 10 shows the restriction enzyme cleavage patterns of clones generated by SalI linker-ligation method

(Section 3.9). These clones are about 1.4-1.6 Kb in length except lane 10 which showed a clone which is 4 Kb in length. Further screening of the transformants are in progress. These colonies will also be hybridized with specific DEN-2 cDNA probes such as pVV1, pVV9 and pVV17 to map the location of the new clones on the dengue virus RNA genome.

2.3.12. cDNA cloning of DEN-2 RNA using a restriction enzyme fragment of pVV17 cDNA (EcoRI + NcoI) as primer.

Experimental. The region between pVV1 and pVV17 needs to be cloned and sequenced. Therefore, a 800 bp fragment released by NcoI + EcoRI was used as primer for reverse transcriptase. The double-stranded cDNA was synthesized as described in the earlier sections. It was cloned using pUC18 as vector and E.coli JM83 as host. Upon transformation of E.coli HB101, 48 colonies were obtained for further characterization.

The restriction enzyme cleavage patterns of four clones are shown in Fig. 12 (a-c). The Southern hybridization data show that the clones hybridize both to pVV1 and pVV17 as probes. Interestingly, the faster migrating band (1.6 Kb in length) hybridizes to pVV1 probe and the slower migrating band (3.6 kb) hybridizes to pVV17 as probe. Therefore, these clones, especially 9-3, hybridizes both to pVV1 and pVV17 map in region II (Fig. 1). Sequence analysis of this clone is in progress.

2.3.13. Cloning of gene coding for NS1 of DEN-2 genome.

Experimental. Using the Primer #1 which we synthesized from our sequence data of pVV9 clone, Dr. Robert Putnak of Walter Reed Army Institute of Research synthesized cDNA from DEN-2 RNA and clone it at the BamH1 site of pUC18 vector and E.coli strain as the host. He characterized five clones pRP2, 17,28,29 and 30 all having identical 5' terminal pVV9 restriction map and in addition, 1.1 kb of DEN-2 region 5' to pVV9 clone, except in pRP2 which contained 1.6 kb of new sequence.

Therefore, these clones contain the complete coding sequence of NS1 and a small portion from the carboxy terminal region of E protein. We are currently sequencing these clones, especially pRP2 and 28, by the chemical method of Maxam and Gilbert.

#### 2.3.14. Mapping of pVV18 DNA.

Experimental. Two DEN-2 clones with fairly long cDNA inserts (1.75 Kb) which were obtained during the previous contract period (DAMD 17-82-C-2051; Annual Report dated March 1986 for contract No. DAMD17-82-C-2051) pVV14 and pVV18 were further characterized to investigate whether they map in the 5'-region coding for structural proteins. A large scale plasmid preparations were carried out. No plasmid DNA was obtained from the clone pVV14 and it might be possible that this particular clone of E.coli has lost its plasmid. However, pVV18 cDNA plasmid was obtained in good yield. Southern hybridization with nick-translated pVV9, pVV1 and pVV17 showed that, it specifically hybridized with pVV9 probe. Digestion with HindIII produced a 450 pb fragment and a 4.0 kb fragment. Digestion with HindIII + BamHI related a 2.7 kb vector, a 1.25 kb and a 450 bp fragments of cDNA. Since pVV9 has a HindIII site at one end of the clone, the 450 bp fragment maps to the 5' end of this HindIII site. Thus, we have a 450 bp fragment which maps in the NS2 regions. Sequence analysis of this fragment is in progress.

#### 2.2.15. Mapping of M13 clones.

Experimental. During the contract period supported by DAMD17-82-C-2051, a number of M13 clones were generated, some of which were sequenced partially using Sanger's dideoxy chain termination method (Progress Report dated September 14, 1983 for Contract DAMD17-82-C-2051). Since specific plasmid cDNA probes are now available, it becomes important to map these M13 clones so that these would be useful for further sequence analysis of dengue-2 RNA. Also these M13 clones would be very useful probes for rapid diagnosis of dengue-2 infections in clinical samples. Single-stranded M13 phage DNA and double-stranded replicative form (RF1) DNAs were prepared from these clones. The single-stranded phage DNAs were

immobilized onto Gene Screen Plus membrane filter and were hybridized with nick-translated HindIII fragment with the NS1 region (5' to the pVV9 clone), pVV9, pVV1, and pVV17 cDNA clones. The results of this hybridization are shown in Table 1. Two M13 clones were found to hybridize specifically to the probe to NS1 region of DEN-2 RNA. One clone was specific for pVV1 and one to pVV17 probes. Currently, the two clones specific to NS1 region are being analyzed for their nucleotide sequence. One of them seems to have a 0.8 kb long insert. It would be possible to use this clone as a probe to "walk" along the DEN-2 RNA genome to map other clones in the 5' end region.

2.3.16. Synthesis of peptides homologous to NS1 and NS5 of DEN-2 RNA.

Experimental.

Based on our sequence analysis of pVV9, pVV1 and pVV17 cDNA clones, synthetic peptides homologous to NS5 and NS1 regions were synthesized. These peptides are being used at Walter Reed Army Institute of Research by Drs. Robert Putnak and Don S. Burke to raise antibodies to study the protein processing of NS1 and NS5 in the DEN-2 infected cells

Peptide #1: H<sub>2</sub>N-KATYEPDVDLGSSTRN-COOH

Peptide #2: H<sub>2</sub>N-EEMLRTRVGTK-COOH

### LEGENDS TO FIGURES

Figure 1 Mapping of DEN-2 cDNA clones with respect to Dengue virus RNA genome. The DEN-2 cDNA clones, pVV1, pVV9, pVV17 and pVV18 were mapped by sequence analysis and by alignment with the prototype flavivirus, yellow fever (YF) virus by using PIR (Georgetown) on-line system facility. The alignment of the putative polyprotein segments to the yellow virus polyprotein showed the location of these DEN-2 cDNA clones on the DEN-2 genome map assuming that YF and DEN-2 have similar genome lengths. I, II, and III represent regions on DEN-2 RNA which need to be cloned and sequenced.

Figure 2 New composite sequence of pVV1 and pVV9. The errors in the sequence presented in Figure 6a in the ANNUAL REPORT #4 (March 1, 1986) for Contract No. DAMD17-82-C-2051 were corrected by sequencing the complementary strand of pVV1 and pVV9 cDNA clones. Only the unambiguous sequence is reported in the manuscript (Yaegashi et al., 1986).

Figure 3 Homologous sequence at the 3' end of YF and WN flavi-viruses. Nucleotide homology between yellow fever virus (YFV) and West Nile virus (WNV). The data are from Rice et al., 1985, for YFV and Wengler and Wengler, 1981 for WNV. The nucleotide identities in the 3' terminal sequences of (+) strand are circled; those between YFV and WN RNA's are underlined. The sequence of the synthetic primer #3 based on the 3'-terminal conserved sequence is shown.

Figure 4 Southern hybridization of synthetic primer #1 with pVV9 cDNA. The sequence, (5') GTAGTCATCATCAAT (3') was synthesized chemically based on our sequence data of pVV9 cDNA clone (see Fig. 2). This sequence (nucleotide #572-586) is complementary to the DEN-2 RNA in the region where pVV9 maps (see Fig. 1). The synthetic DNA primer was purified as described in the text and was labeled as the 5' end using polynucleotide kinase and ( $\gamma$ -<sup>32</sup>P) ATP. The labeled DNA primer was used as a probe for Southern hybridization with the PstI digest of pVV17 (4a & 4b, lane 1) and pVV1 (4a & 4b, lane 2) plasmids, fractionated by electrophoresis on an agarose gel (1%).

Fig. 4b is the photograph of the gel stained with ethidium bromide and 4a is the autoradiograph of the gel subsequent to the transfer to Gene Screen Plus membrane filter and hybridization with the probe (Primer #1). Probe is seen to hybridize specifically to the 1.6 kb cDNA insert released from pVV9 plasmid (Fig. 4a, lane 2), as expected.

**Figure 5** Southern hybridization of synthetic primer #2 with pVV17 cDNA. The sequence (5') ACCCCTTCCGCTCATC (3') was synthesized based on our sequence data of pVV17 cDNA clone (see Fig. 6b of ANNUAL REPORT dated March 1986 for Contract No. DAMD 17-82-C-2051). This sequence (nucleotide #141-1156) is complementary to the DEN-2 RNA in the region where pVV17 maps (see Fig. 1). The synthetic DNA primer was purified as described in the text and was labeled at the 5' end using polynucleotide kinase and ( $\gamma$ -<sup>32</sup>P) ATP. The labeled DNA primer was used as probe for Southern hybridization with the Pst I digest of both pVV17 (Fig. 5a & 5b, lane 1) and pVV9 (Fig. 5a & 5b, lane 2) plasmids, fractionated by electrophoresis on an agarose gel (1%). Fig. 5b is the photograph of the gel stained with ethidium bromide and 5a is the autoradiograph of the gel subsequent to the transfer to Gene Screen Plus membrane filter and hybridization with the probe (Primer #2). Probe is seen to hybridize specifically to the 2.0 kb cDNA insert released from pVV17 plasmid (Fig. 5a, lane 1), as expected.

**Figure 6** Strategy for cloning cDNA from DEN-2 RNA template. The DEN-2 RNA template and random calf thymus DNA primers (Taylor et al., 1976; Rice et al., 1981) were used for the first strand synthesis by reverse transcriptase. The second strand synthesis was carried out according to Gubler and Hoffman (1983). The double-stranded cDNA library thus obtained was used for cloning in three separate pathways. In the first, it was oligo(dC)-tailed and annealed with oligo(dG)-tailed pUC18 vector DNA. In the second, it was ligated to synthetic SalI linker, was cleaved with SalII and was purified by agarose gel electrophoresis. In the third, it was treated with EcoRI DNA methylase, followed by ligation to EcoRI linkers and cleavage by EcoRI. After ligation

of the cDNA library with the vector DNA, appropriately cleaved and dephosphorylated (for example, pUC18 DNA cut by SalI in Exp.#2 and EcoRI in Exp.#3 before dephosphorylation), the recombinant plasmids was used for transformation of E.coli JM83 and the transformants were plated on LB agar plates containing 50 µg/ml ampicillin, and 40 µg/ml each of IPTG and X-gal. The white colonies were picked and screened for cDNA inserts by restriction enzyme analysis and Southern hybridization.

Figure 7 Size analysis of double-stranded cDNA by random calf thymus priming. The size of the cDNA library was determined by alkaline agarose gel electrophoresis. The autoradiograph is shown. The size of the cDNA varied from 500 bp to > 2 kb in length.

Figure 8 Restriction patterns and Southern hybridization of cDNA clones generated by olig(dC)-tailing method. The plasmid DNAs from clones generated from oligo(dC)-tailing method were prepared by alkaline lysis method (Maniatis et al., 1982). The DNAs were cut by PstI and the digests were fractionated on an agarose gel (1%). The gel was stained by ethidium bromide and photographed (Fig. 8a). Lane 1-18 contained  $\lambda$ DNA + HindIII digest as DNA markers and 16 clones generated by method 1 shown in Fig. 6. After transfer to Gene Screen Plus membrane filter, the filter was hybridized with the cDNA probe prepared from DEN-2RNA using random primers. Only clones B3 (lane 4), B7 (lane 5), C-11 (lane 7), D4 (lane 8), D-10 (lane 9) G5 (lane 10), G-11 (lane 13), G12 (lane 14), H8 (lane 15), H9 (lane 16), and H11 (lane 17) were found to hybridize with the probe. Out of 27 clones picked randomly, 14 clones were found to contain DEN-2 cDNA inserts (see Figure 9). Many more clones remain to be screened by this approach.

Figure 9 Restriction patterns and Southern hybridizations of cDNA clones generated by oligo(dC)-tailing and EcoRI linker ligation. The plasmid DNAs from clones generated from oligo(dC)-tailing as well as EcoRI linker ligation (see Fig. 6) were analyzed by restriction with PstI (lanes 2-12) or EcoRI (lane 13). The digests were fractionated by agarose gel electrophoresis (1%) and the gel was photographed after staining with

ethidium bromide (Fig. 9a). The DNA bands were transferred to Gene Screen Plus and hybridized with the probe as described under Figure 8. Only clones A4 (lane 4), B1 (lane 7), and B5 (lane 6) obtained by oligo(dC)-tailing method and the clone #22 (lane 12) were found to hybridize with the DEN-2 cDNA probe.

**Figure 10** Restriction patterns and Southern hybridizations of cDNA clones generated by SalI linker ligation. The plasmid DNAs from clones generated from ligation of synthetic SalI linkers to the ds cDNA followed by cleavage with SalI to produce 5' protruding ends and cloning at the SalI site of pUC18 DNA/E.coli JM83 host were purified as described in Figs. 8 and 9. They were analyzed by cleavage with SalI and agarose gel electrophoresis. Fig. 10a shows the ethidium bromide stained gel and 10b shows the autoradiograph of the gel after hybridization with the cDNA probe. DNAs from clones 6, 10, 20, 25, 32, 62, 63, 78, 101, 102, 105 were found to be positive in hybridization and were loaded in lanes 2, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14, respectively. Many other clones remain to be screened in this manner.

**Figure 11** Restriction enzyme cleavage pattern and Southern hybridization of clones generated by specific primer to pVV17 region. Using the EcoRI-NcoI cleaved restriction fragment of pVV17 clone as the primer for reverse transcriptase and oligo(dC)-tailing method for insertion into the PstI site of pUC13'-1 vector, several transformants were obtained. Restriction analysis of five such clones (lanes 2-6) by double digestion with BamHI + HindIII are shown in Fig. 11a. Lane 1 contained the DNA markers ( $\lambda$  DNA + HindIII). Autoradiographs of such a gel transferred to Gene Screen Plus membrane filter and hybridized with pVV1 cDNA (Fig. 11b) and pVV17 cDNA (Fig. 11c) are shown. Upon alignment of the autoradiograph with the membrane filter, it was found that, the pVV1 cDNA probe hybridized with the lower band (1.7 kb) (Fig. 11b) and pVV17 cDNA probe hybridized with the upper band (3.7 kb) (Fig. 11c), indicating that the cDNA in these plasmid clones pSV 1-5, comes from the region II of Dengue RNA (see Fig. 1) and that

BamHI + HindIII cleaves in such a manner that pVV1 specific portion is released from the vector and the pVV17 specific portion is still attached to the vector (2.7 kb vector + 1 kb insert).

Fig. 1

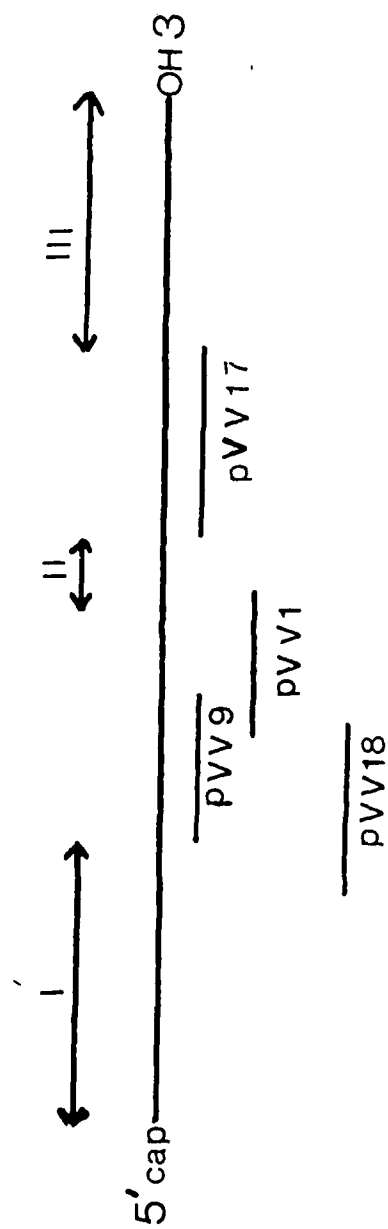


FIGURE 2

2677  
885

Fig. 3

YFV

..... AACACACAAAACCACU 3'  
..... UUUGUGUUUUGGUGA 5'

WNV

..... AACACAGGAUCU 3'  
..... UUUGUCCUAGA 5'

Primer # 3

AGTGGTTTGTGTT

Fig.4

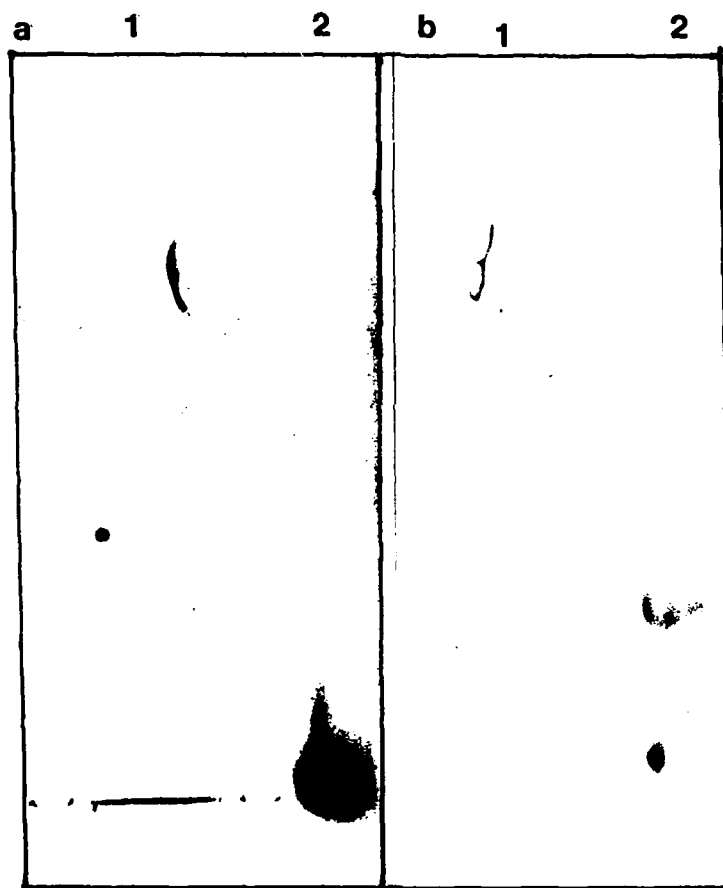
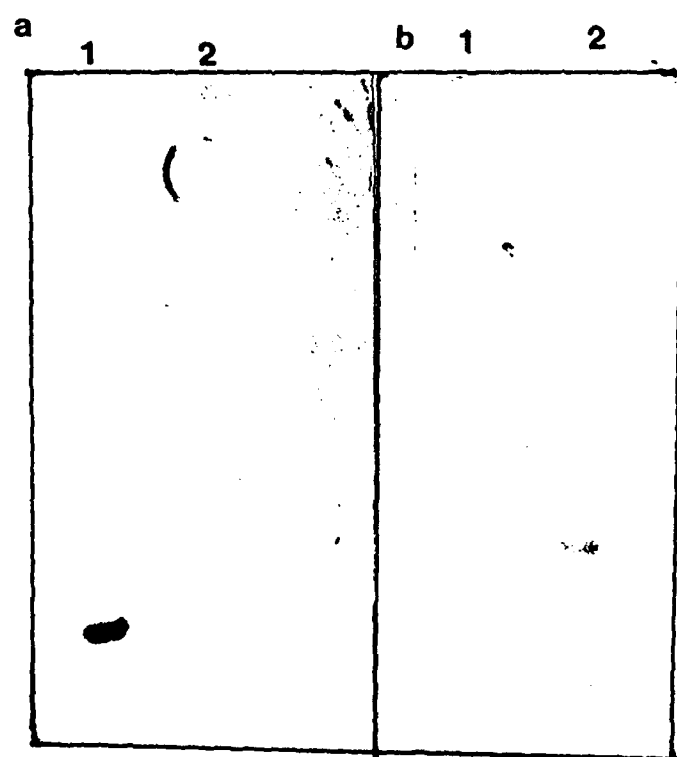


Fig.5



**Fig.6**

DEN-2 RNA

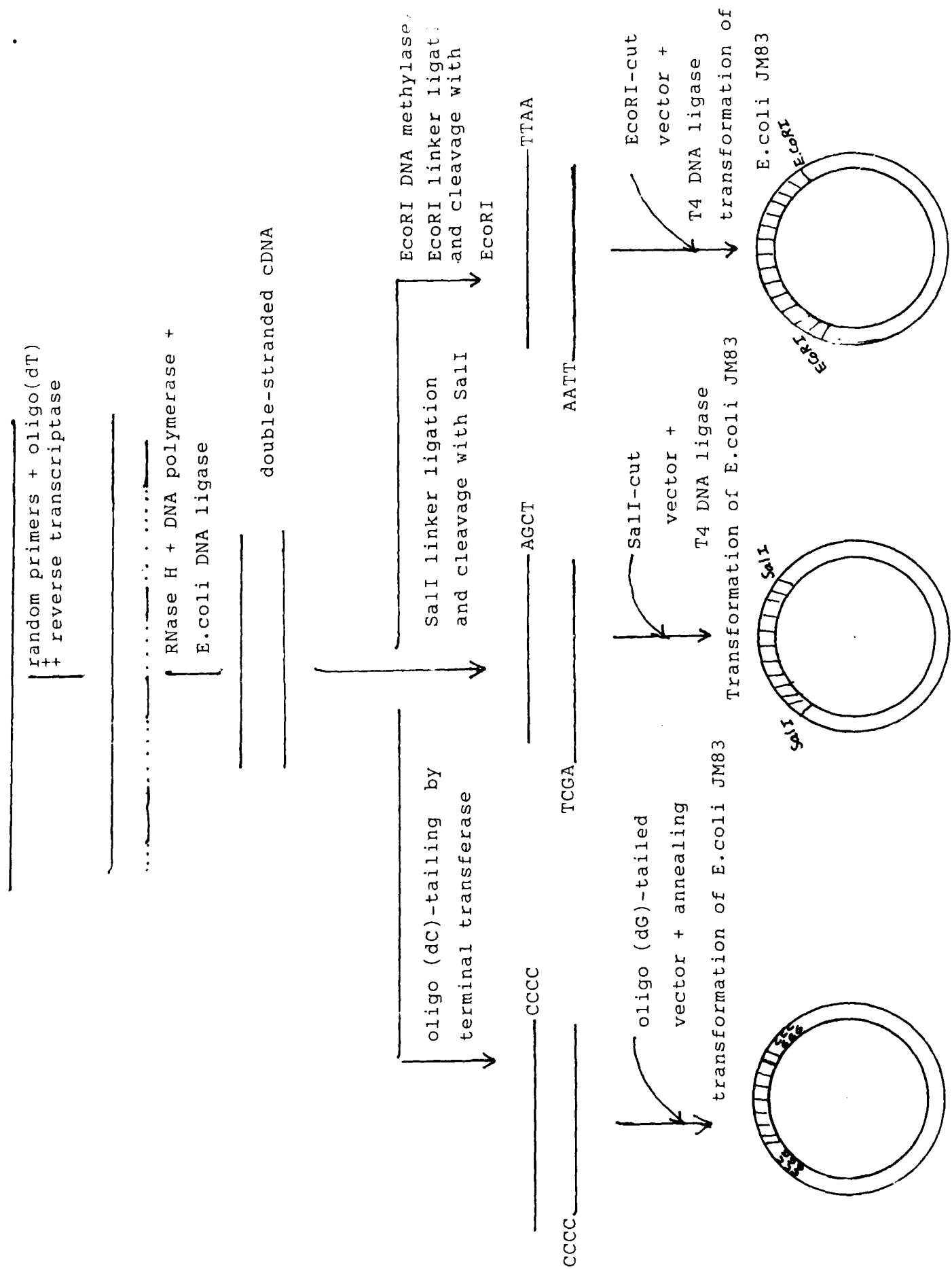


Fig.7

$\lambda$   $\rightarrow$  2kb

500  $\rightarrow$  0.5kb



Fig.8

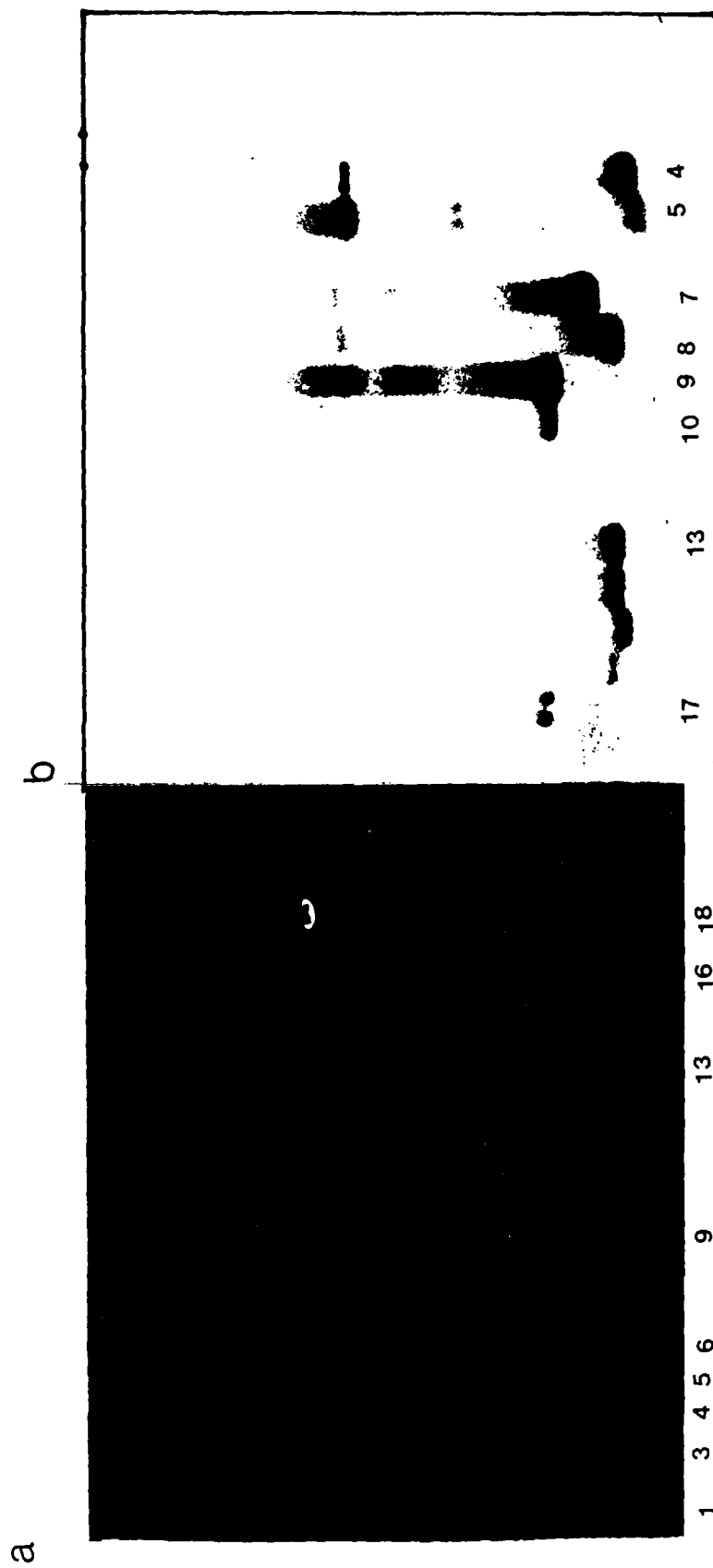


Fig.9

a

b

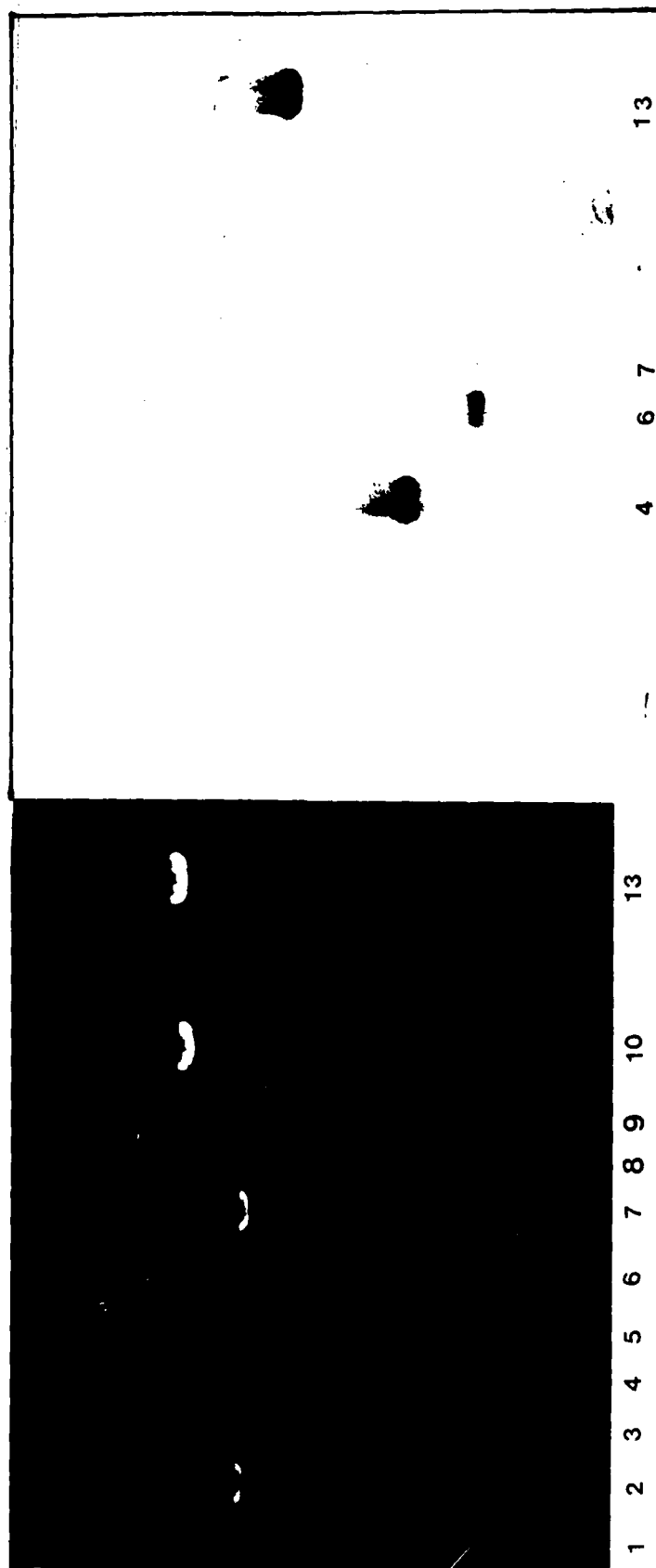


Fig. 10

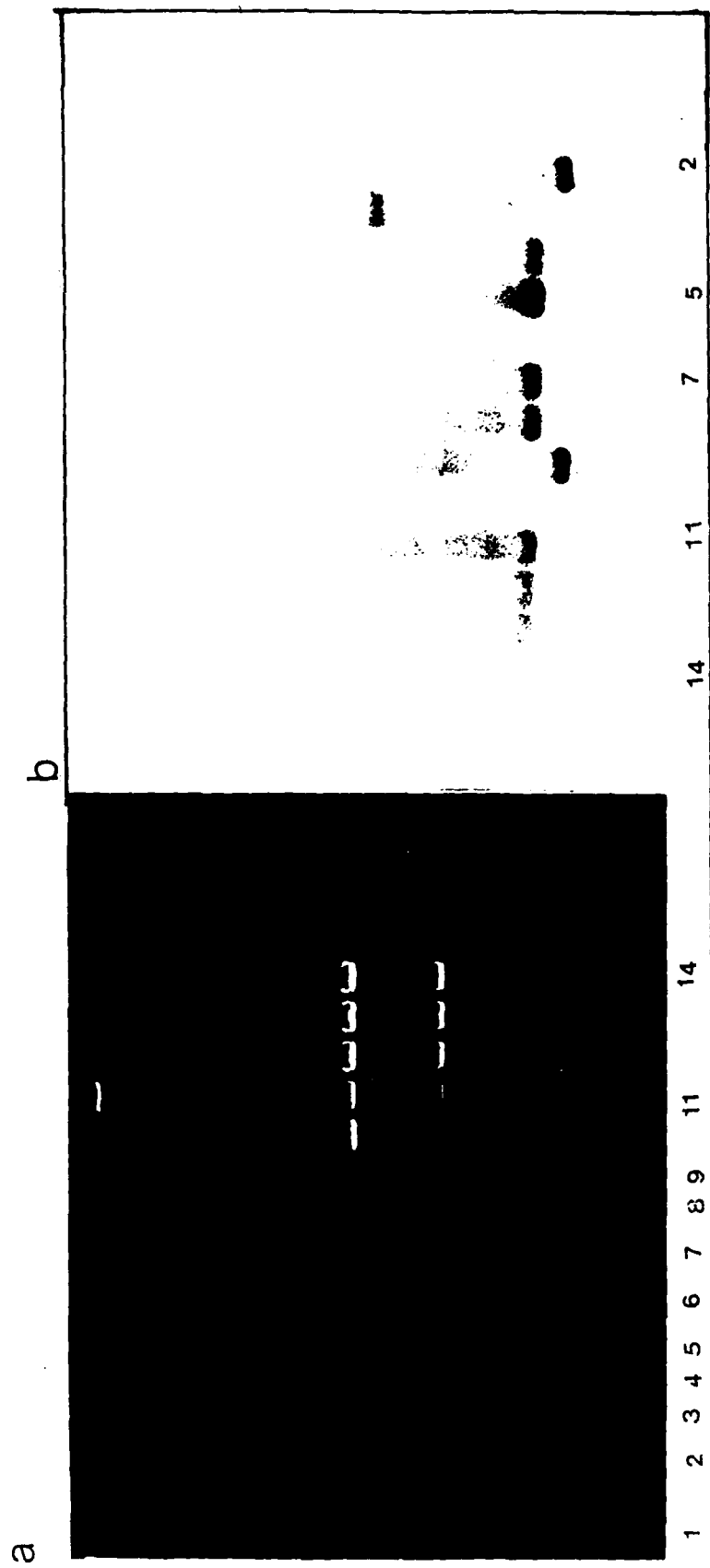
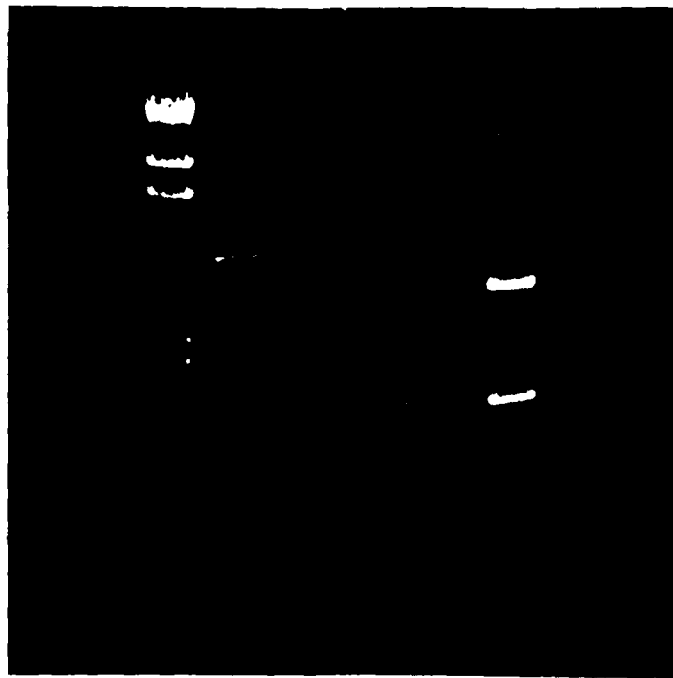


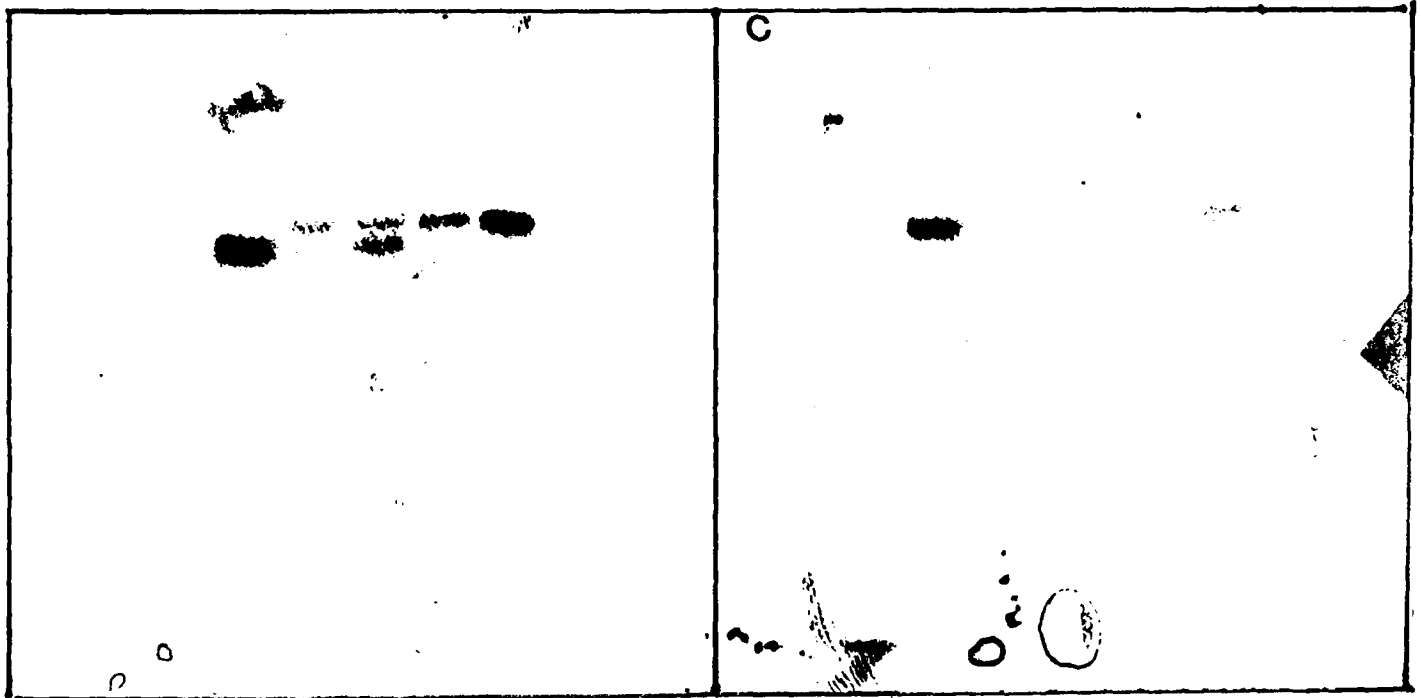
Fig.11.

a



← 3.7kb

← 1.7kb



# Table I

Hybridization of pVV18-Hind III-B, pVV1  
and pVV17 cDNA probes to M13 clones

<u>M13</u>	<u>Probe</u>	<u>M13</u>	<u>Probe</u>	<u>M13</u>	<u>Probe</u>
I-6		I-7		I-8	
I-12		I-16		I-17	
I-20		I-24		I-102*	pVV18 HindIII-B
I-103*	pVV18 HindIII-B				
II-1		II-9*	pVV17	II-18	
213		223		224	
255		258		267	
273		280		281	
290					

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Personnel supported from September 15, 1985 - September 14, 1986

<u>Personnel</u>	<u>% Effort</u>	<u>Months</u>	<u>Period</u>
<u>Research Associates</u>			
Dr. Nai-Zheng Xu	100	6	Jan. 2, 1986 - June 30, 1986
Dr. Fu-Rong Shang	100	8	Dec. 1985 - July 31, 1986
Dr. Yasuyuki Sasaguri	100	4.5	May 1, 1986 - present
Dr. Gunwar Sripad	100	8	Jan. 15, 1986 - Sept. 14, 1986
		----	
Total Man-months		26.5	
		----	
<u>Research Assistants</u>			
Nasima Alam (worked at WRAIR)	100	10	Sept. 15, 1985 - July 18, 1986
Nashaud Merchant	100	5	Dec. 1985 - April 30, 1986
Dianne Vassmer	100	1.5	August 1, 1986 - present
		----	
Total Man-months		16.5	
<u>Graduate students</u>			
Thaweesak Trirawatanapong	50	6	Sept. 15, 1985 - present
<u>Laboratory aide</u>			
Mike Sullivan	50	5	Sept. 15, 1985 - Aug. 8, 1986
<u>Principal Investigator</u>			
Radha R. Padmanabhan	35	4.2	Sept. 15, 1985 - present

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